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A Single-Base Change within the DNA Polymerase Locus of Herpes Simplex Virus Type 2 Can Confer Resistance to Aphidicolin

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An aphidicolin-resistant (Aph^r) mutant of herpes simplex virus (HSV) type 2 strain 186 previously has been shown to induce an altered viral DNA polymerase that is more resistant to aphidicolin and more sensitive to phosphonoacetic acid (PAA) than is wild-type DNA polymerase. In this study the mutation responsible for the aphidicolin-resistant phenotype was physically mapped by marker transfer experiments. The physical map limits for the Aph^r mutation were contained in a 1.1-kilobase pair region within the HSV DNA polymerase locus. The 1.1-kilobase-pair fragment of the Aph^r mutant also conferred hypersensitivity to PAA, and DNA sequence analysis revealed an AT to GC transition within this fragment of the Aph^r mutant. Analysis of the three potential open reading frames within the 1,147-base-pair fragment and comparison with the amino acid sequence of DNA polymerase of HSV type 1 indicated that the Aph^r mutant polymerase had an amino acid substitution from a tyrosine to a histidine in the well-conserved region of the DNA polymerase. These results indicate that this single amino acid change can confer altered sensitivity to aphidicolin and PAA and suggest that this region may form a domain that contains the binding sites for substrates, PP$_i$, and aphidicolin.

Herpes simplex virus (HSV) induces a novel DNA polymerase in infected cells that is immunologically and biochemically distinct from host cell DNA polymerase (18, 32). The viral DNA polymerase has been purified and extensively characterized with respect to its substrate specificity, reaction optima, and kinetic behavior (18, 24, 32). The major component of purified polymerase is a polypeptide with a molecular weight of about 140,000 that bears DNA-polymerizing activity (18, 24). To map the HSV DNA polymerase gene in the viral genome, a variety of temperature-sensitive or drug-resistant mutants have been isolated (4, 7–12, 16, 17), and the viral DNA polymerase gene has been located in the region 0.413 to 0.434 on the physical map of HSV type 1 (HSV-1) strain KOS (3, 7, 8, 11). Recently, the DNA sequences of this region of HSV-1 have been determined by Gibbs et al. (15) and Quinn and McGeoch (25). The region contained the gene which included a 3,705-base-pair (bp) open reading frame that is capable of encoding a polypeptide with a molecular weight of approximately 137,000, which is in agreement with the previous estimated size of the major polypeptide of viral DNA polymerase.

Aphidicolin, a tetracyclic diterpenoid, effectively inhibits the activity of HSV DNA polymerase in vitro as well as the replication of HSV in cultures (13, 22–24), although this compound has been known to be a highly specific inhibitor of eucaryotic α-type DNA polymerase (13). We have recently isolated an aphidicolin-resistant (Aph^r) mutant from HSV type 2 (HSV-2) using aphidicolin as the selective agent, and have shown that the mutant induces an altered viral DNA polymerase that has a reduced affinity to aphidicolin and an increased affinity to dCTP and dTTP compared with that of the parental wild type (22). In addition, the Aph^r mutant exhibited hypersensitivity to a PP$_i$ analog, phosphonoacetic acid (PAA). Thus, it may be reasonable to expect that the mutation occurs within or adjacent to the binding sites for aphidicolin, PP$_i$, and substrates.

In this study we show by marker transfer experiments that a 1.1-kilobase-pair (kbp) region within the HSV DNA polymerase locus contains a mutation that confers altered sensitivity to aphidicolin. Furthermore, we compared the nucleotide sequences of this portion between Aph^r and wild-type viruses and determined the mutation site that is responsible for the aphidicolin resistance phenotype.

MATERIALS AND METHODS

Cells and virus. African green monkey kidney cells (Vero) were grown in Eagle minimal essential medium supplemented with 5% calf serum, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Human embryonic fibroblasts were prepared as described previously (20) and used for the assay of plating efficiency of viruses. Wild-type HSV-2 strain 186 was originally obtained from Fred Rapp, Pennsylvania State University College of Medicine, University Park, Pa. (21). Isolation of the Aph^r mutant has been described previously (22).

Chemicals and enzymes. Aphidicolin and disodium PAA were purchased from Wako Pure Chemicals and Sigma Chemical Co. (St. Louis, Mo.), respectively. Restriction endonucleases were purchased from Wako Pure Chemicals and Toyobo Co., Ltd., and used under conditions recommended by the suppliers. T4 DNA ligase and bacterial alkaline phosphatase were obtained from Takara Shuzo Co., Ltd. The Klenow fragment of *Escherichia coli* DNA polymerase I, DNA sequencing reagents, and [α-^{32}P]dCTP (400 Ci/mmol) were obtained from Amersham Corp. (Arlington Heights, Ill.).

Preparation of DNA. Infectious HSV DNA to be used for marker transfer experiments was prepared as follows. Wild-type HSV-2 strain 186 was infected onto monolayers of Vero cells at a multiplicity of approximately 0.1 PFU per cell. After incubation for 20 h at 36°C, the medium was collected

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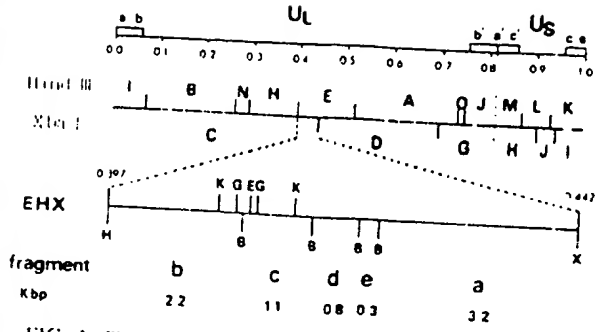


FIG. 1. Physical map of the HSV-2 strain 186 DNA molecule. The top line is a schematic representation of the HSV-2 DNA molecule with physical map coordinates. The second line represents recognition sites for *Hin*dIII and *Xba*I endonucleases on DNA isolated from HSV-2 strain 186. The third line shows an expanded region (EHX) between coordinates 0.397 and 0.442 which contains the *pol* locus, with restriction endonuclease recognition sites for *Bam*HI (B), *Bgl*II (G), *Hin*dIII (H), *Eco*RI (E), *Kpn*I (K), and *Xba*I (X). Sizes of the fragments (a, b, c, d, and e) generated by digestion of the EHX fragment with *Bam*HI endonuclease are depicted at the bottom of the figure. Abbreviations: U$_L$, long unique sequences; U$_S$, short unique sequences; a, b, and c, terminal and internal redundant sequences.

and centrifuged at 3,000 × *g* for 10 min. The supernatant was further centrifuged at 87,700 × *g* for 1 h at 4°C to make a pellet of extracellular viruses. The pellet was suspended in TNE buffer (20 mM Tris hydrochloride [pH 7.4], 150 mM NaCl, 1 mM EDTA), layered onto a linear 10 to 50% sucrose gradient, and centrifuged at 74,700 × *g* for 1 h. The virus band was collected and pelleted at 87,700 × *g* for 1 h. The pellet was suspended in TNE buffer and then treated with proteinase K (200 μg/ml) and 1% sodium dodecyl sulfate. After extraction twice with phenol-chloroform, the viral DNA was dialyzed for 24 h at 4°C against TE buffer (10 mM Tris hydrochloride [pH 7.4], 1 mM EDTA). Plasmids pAE-1 and pAH-1, which contained *Hin*dIII E and *Hin*dIII H fragments of Aph^r viral DNA, respectively, were obtained from a shotgun cloning experiment by previously described methods (30). The identification of the cloned recombinant plasmids containing *Hin*dIII E and H fragments was performed by Southern blot hybridization analysis of ^{32}P-labeled, individually cloned recombinant plasmids to HSV-2 DNA digested with *Hin*dIII or *Eco*RI and by double digestion analysis of cloned DNA fragments with *Hin*dIII-*Hpa*I, *Hin*dIII-*Xba*I, or *Hin*dIII-*Eco*RI (data not shown). Plasmid pAEHX containing the EHX fragment (Fig. 1) was obtained from plasmid pAE-1 by digestion with *Xba*I and self-ligation. Digestion of the EHX fragment with *Bam*HI generated five DNA fragments: a, b, c, d, and e. These fragments were separated on a vertical 0.7% agarose slab gel cast in 40 mM Tris–5 mM sodium acetate–1 mM EDTA, isolated by the glass bead method (31), cloned into pACYC184 (2), and then propagated in *Escherichia coli* HB101.

Marker transfer. Marker transfer experiments were performed by transfecting Vero cells with a mixture of intact wild-type DNA and individual Aph^r DNA fragments cloned into pACYC184. Plasmid DNAs used for marker transfer

TABLE 1. Effect of aphidicolin and PAA on the plating efficiencies of wild-type, Aph^r mutant, and progeny Aph^r mutant viruses

Virus	Plating efficiency with[a]:	
	Aphidicolin (2 μg/ml)	PAA (25 μg/ml)
Wild type	<0.05	0.60
Aph^r mutant	0.83	<0.05
Progeny Aph^r	0.75	<0.05

[a] To determine the plating efficiency, confluent monolayers of human embryonic fibroblasts were infected with approximately 100 PFU of each virus. After a 1-h adsorption period, the cultures were overlaid with 0.5% agarose in Eagle minimal essential medium containing 2 μg of aphidicolin per ml, 25 μg of PAA per ml, or neither compound. The number of plaques was counted at 2 days postinfection, as described previously (22).

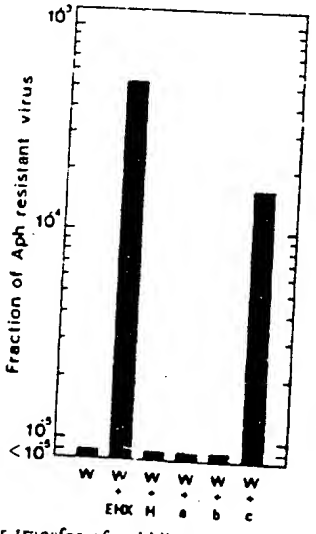


FIG. 2. Marker transfer of aphidicolin resistance phenotype of the Aph^r mutant virus. Vero cells were cotransfected with cloned DNA fragments of the Aph^r mutant and infectious wild-type (W) DNA of HSV-2 strain 186. The progeny were then assayed for resistance to aphidicolin by measuring the plating efficiency on human embryonic fibroblast monolayers in the presence of 5 μg of aphidicolin per ml. H indicates the *Hin*dIII H fragment of Aph^r EHX. a, b, and c are defined in the legend to Fig. 1.

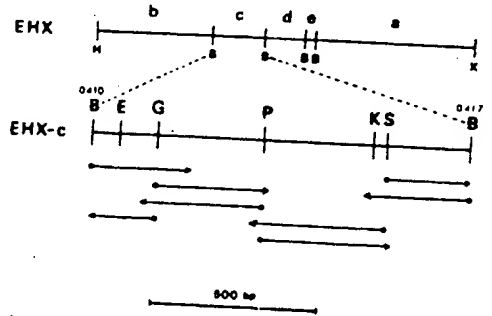
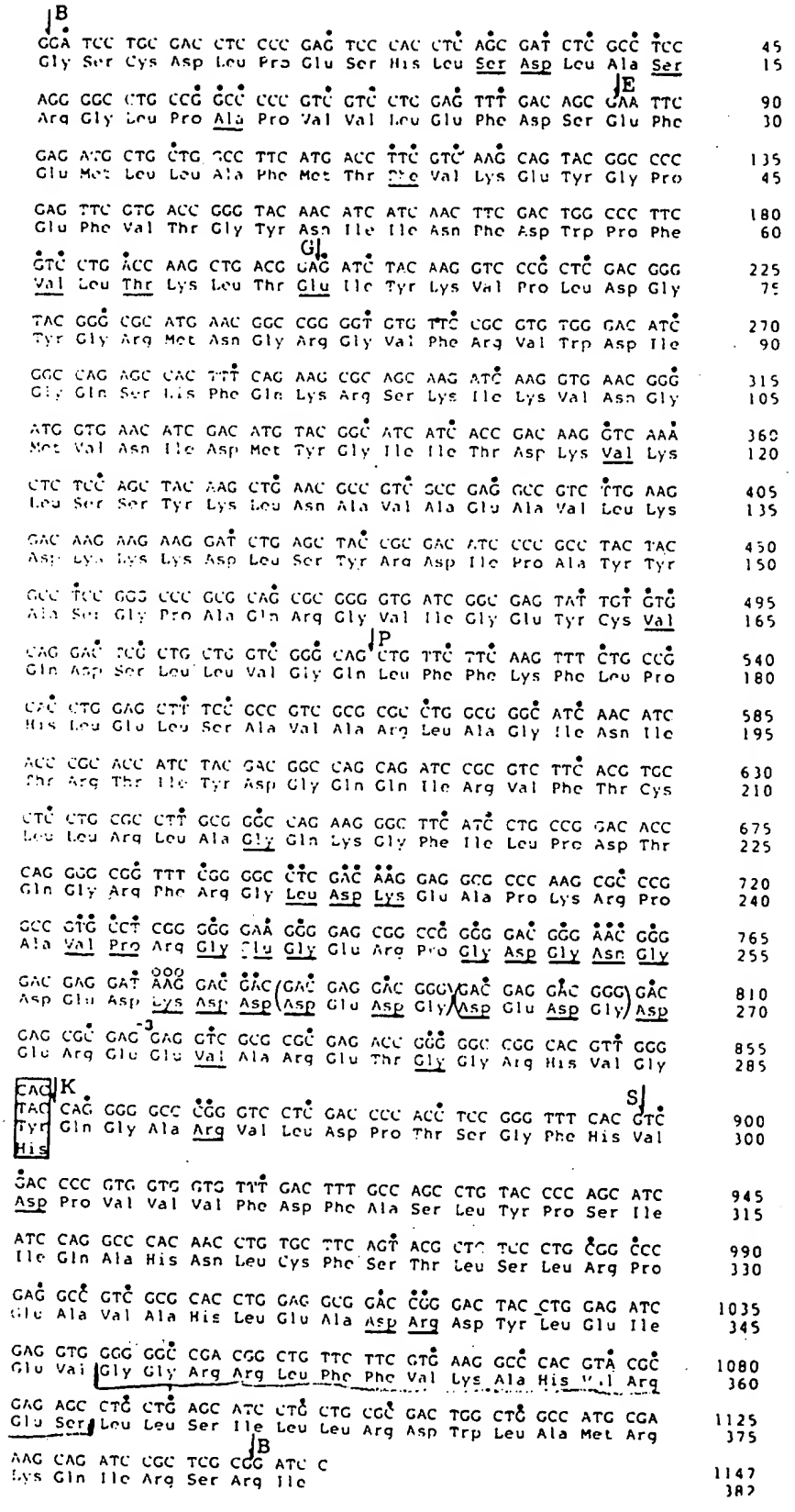


FIG. 3. Restriction site map and strategy for determination of the DNA sequence of the EHX-c fragment of wild-type or Aph^r mutant viruses. Arrows indicate the direction and extent of sequence determination. Endpoints for sequence analyses are indicated with solid circles (5' ends) and arrowheads (3' ends). The positions of several restriction endonuclease sites are also indicated. Restriction enzyme abbreviations are as follows: B, *Bam*HI; E, *Eco*RI; G, *Bgl*II; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sal*I; X, *Xba*I.


B
GGA TCC TGC GAC CTC CCC GAG TCC CAC CTC AGC GAT CTC GCC TCC 45
Gly Ser Cys Asp Leu Pro Glu Ser His Leu Ser Asp Leu Ala Ser 15
E
AGG GGC CTG CCG GCC CCC GTC GTC CTC GAG TTT GAC AGC GAA TTC 90
Arg Gly Leu Pro Ala Pro Val Val Leu Glu Phe Asp Ser Glu Phe 30
GAG ATG CTG CTG GCC TTC ATG ACC TTC GTC AAG CAG TAC GGC CCC 135
Glu Met Leu Leu Ala Phe Met Thr Phe Val Lys Glu Tyr Gly Pro 45
GAG TTC GTG ACC GGG TAC AAC ATC ATC AAC TTC GAC TGG CCC TTC 180
Glu Phe Val Thr Gly Tyr Asn Ile Ile Asn Phe Asp Trp Pro Phe 60
G
GTC CTG ACC AAG CTG ACG GAG ATC TAC AAG GTC CCG CTC GAC GGG 225
Val Leu Thr Lys Leu Thr Glu Ile Tyr Lys Val Pro Leu Asp Gly 75
TAC GGG CGC ATG AAC GGC CGG GGT GTG TTC CGC GTG TGG GAC ATC 270
Tyr Gly Arg Met Asn Gly Arg Gly Val Phe Arg Val Trp Asp Ile 90
GGC CAG AGC CAC TTT CAG AAG CGC AGC AAG ATC AAG GTG AAC GGG 315
Gly Gln Ser His Phe Gln Lys Arg Ser Lys Ile Lys Val Asn Gly 105
ATG GTG AAC ATC GAC ATG TAC GGC ATC ATC ACC GAC AAG GTC AAA 360
Met Val Asn Ile Asp Met Tyr Gly Ile Ile Thr Asp Lys Val Lys 120
CTC TCC AGC TAC AAG CTG AAC GCC GTC GCC GAG GCC GTC TTG AAG 405
Leu Ser Ser Tyr Lys Leu Asn Ala Val Ala Glu Ala Val Leu Lys 135
GAC AAG AAG AAG GAT CTG AGC TAC CGC GAC ATC CCC GCC TAC TAC 450
Asp Lys Lys Lys Asp Leu Ser Tyr Arg Asp Ile Pro Ala Tyr Tyr 150
GCC TCC GGG CCC GCG CAG CGC GGG GTG ATC GGC GAG TAT TGT GTG 495
Ala Ser Gly Pro Ala Gln Arg Gly Val Ile Gly Glu Tyr Cys Val 165
P
GAG GAC TCG CTG CTG GTC GGG CAG CTG TTC TTC AAG TTT CTG CCG 540
Gln Asp Ser Leu Leu Val Gly Gln Leu Phe Phe Lys Phe Leu Pro 180
CAC CTG GAG CTT TCC GCC GTC GCG CGC CTG GCG GGC ATC AAC ATC 585
His Leu Glu Leu Ser Ala Val Ala Arg Leu Ala Gly Ile Asn Ile 195
ACC CGC ACC ATC TAC GAC GGC CAG CAG ATC CGC GTC TTC ACG TGC 630
Thr Arg Thr Ile Tyr Asp Gly Gln Gln Ile Arg Val Phe Thr Cys 210
CTC CTG CGC CTT GCG GGC CAG AAG GGC TTC ATC CTG CCG GAC ACC 675
Leu Leu Arg Leu Ala Gly Gln Lys Gly Phe Ile Leu Pro Asp Thr 225
CAG GGC CGG TTT CGG GGC CTC GAC AAG GAG GCG CCC AAG CGC CCG 720
Gln Gly Arg Phe Arg Gly Leu Asp Lys Glu Ala Pro Lys Arg Pro 240
GCC GTG CCT CGG GGG GAA GGG GAG CGG CCG GGG GAC GGG AAC GGG 765
Ala Val Pro Arg Gly Glu Gly Glu Arg Pro Gly Asp Gly Asn Gly 255
GAC GAG GAT AAG GAC GAC GAC GAC GAG GAC GGG GAC GAG GAC GGG GAC 810
Asp Glu Asp Lys Asp Asp Asp Glu Asp Gly Asp Glu Asp Gly Asp 270
GAG CGC GAC GAG GTC GCG CGC GAG ACC GGG GGC CGG CAC GTT GGG 855
Glu Arg Glu Glu Val Ala Arg Glu Thr Gly Gly Arg His Val Gly 285
CAG K
TAC CAG GGG GCC CGG GTC CTC GAC CCC ACC TCC GGG TTT CAC GTC 900
Tyr Gln Gly Ala Arg Val Leu Asp Pro Thr Ser Gly Phe His Val 300
His
S
GAC CCC GTG GTG GTG TTT GAC TTT GCC AGC CTG TAC CCC AGC ATC 945
Asp Pro Val Val Val Phe Asp Phe Ala Ser Leu Tyr Pro Ser Ile 315
ATC CAG GCC CAC AAC CTG TGC TTC AGT ACG CTC TCC CTG CGG CCC 990
Ile Gln Ala His Asn Leu Cys Phe Ser Thr Leu Ser Leu Arg Pro 330
GAG GCC GTC GCG CAC CTG GAG GCG GAC CGG GAC TAC CTG GAG ATC 1035
Glu Ala Val Ala His Leu Glu Ala Asp Arg Asp Tyr Leu Glu Ile 345
GAG GTG GGG GGC CGA CGG CTG TTC TTC GTG AAG GCC CAC GTA CGC 1080
Glu Val Gly Gly Arg Arg Leu Phe Phe Val Lys Ala His Val Arg 360
GAG AGC CTC CTG AGC ATC CTG CTG CGC GAC TGG CTG GCC ATG CGA 1125
Glu Ser Leu Leu Ser Ile Leu Leu Arg Asp Trp Leu Ala Met Arg 375
B
AAG CAG ATC CGC TCG CGG ATC C 1147
Lys Gln Ile Arg Ser Arg Ile 382

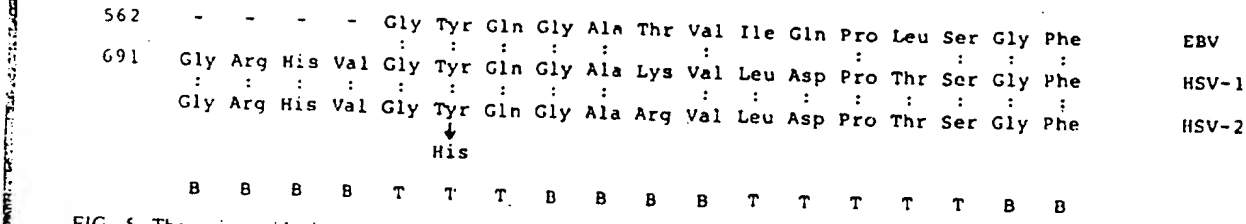


FIG. 5. The amino acid alignment around the mutation site among EBV, HSV-1, and HSV-2 DNA polymerase protein sequences and the predicted secondary structure for the region of HSV-2. The beginning position of each protein is given in the left margin. Identical residues are marked as with colons. Dashes represent gaps that were introduced for alignment. The amino acid sequences of HSV-1 and EBV DNA polymerases are from Gibbs et al. (15) and Baer et al. (1), respectively. The predicted secondary structure for the region of HSV-2 is shown. Abbreviations: B, β-sheet; T, reverse turn.

were cleaved free of the vector or linearized by digestion with appropriate restriction enzymes, because this procedure has been shown to increase recombination efficiency (8). The specific infectivity of wild-type DNA was usually 200 to 300 PFU/μg of viral DNA. Vero cells that were at subconfluency in 25-cm² flasks were transfected with 1 ml of a calcium phosphate precipitate containing 13 μg of salmon sperm DNA, 2 μg of intact wild-type DNA, and 5 μg of restriction enzyme-digested plasmid DNA derived from the Aph' mutant. The molar ratio of intact DNA to DNA fragments was varied from 1:25 to 1:350. Cells were exposed to the mixed DNA for 5 h and then shocked with 20% (vol/vol) dimethyl sulfoxide for 1 min to increase the efficiency of transfection (29). The cultures were incubated at 37°C until total cytopathic effect was observed.

In 5 μg of aphidicolin per ml, the plating efficiency of the Aph' mutant was more than 1,000-fold higher than that of wild-type virus. Therefore, the progeny viruses from marker transfer experiments were tested for their ability to form plaques in 5 μg of aphidicolin per ml.

DNA sequence analysis. The 1.1-kbp EHX-c fragments (Fig. 1) were derived from both aphicidolin-resistant and wild-type DNA and subcloned into *Bam*HI-restricted pACYC184. Defined restriction fragments derived from EHX-c DNA were inserted into the cloning and sequening vectors M13 mp10, mp11, or mp19 (27). Following ligation and transformation of JM109 cells, the progeny M13 phages were plated under selective conditions, and putative recombinants were picked. Sequence analysis by the dideoxynucleotide triphosphate chain termination method (26) was performed with single-stranded M13 phage DNAs as a template. The synthesis reactions were primed with a 17-residue fragment (TAKARA Shuzo Co., Ltd.) that hybridized adjacent to the inserted DNA. Reactions were extended with Klenow DNA polymerase in the presence of [^{32}P]dCTP. Thin sequencing gels containing 6% polyacrylamide were used to resolve the products. The dried gels were exposed on Fuji XR film overnight.

RESULTS

Marker transfer of the aphidicolin-resistant phenotype. Physical restriction endonuclease mapping of the 7.6-kbp EHX fragment was performed by single and multiple enzyme digestions of plasmid pWEHX (Fig. 1). Restriction enzyme patterns of HSV-2 strain 186 in this region were almost identical with those of strain HG52 (4), except that the former had additional cleavage sites to restriction endonucleases *Bam*HI and *Bgl*II. It was also noted that a cleavage site to *Kpn*I was lost in the EHX-c fragment of the Aph' mutant DNA.

Definition of the location of the aphidicolin resistance mutation was accomplished by the marker transfer approach. Marker transfer experiments were performed by transfecting Vero cells with a mixture of intact wild-type DNA and individual Aph' DNA fragments cloned into pACYC184, and the progeny viruses were tested for their ability to form plaques in 5 μg of aphidicolin per ml. In the initial approach, large fragments of Aph' mutant DNA such as *Hin*dIII-H and EHX were employed for transfer to obtain a preliminary localization of the mutation. Results of these marker transfer experiments suggest that the mutation is localized on the DNA polymerase locus, not on the major DNA-binding protein locus. The next experiments were performed with cloned DNA fragments that were derived from the EHX fragment of the Aph' mutant. The EHX-c fragment of the Aph' mutant transferred aphidicolin resistance 16-fold more efficiently than when no fragment was added, whereas the EHX-a and -b fragments did not (Fig. 2). The progeny that acquired aphidicolin resistance in these transfections were also tested for sensitivity to PAA. While the plating efficiency of wild-type virus was 0.60 with 25 μg of PAA per ml, those of the Aph' mutant and the progeny Aph' mutant viruses were less than 0.05 (Table 1). These results indicate that the EHX-c fragment confers altered sensitivity to both aphidicolin and PAA.

Comparative nucleotide sequence analysis of DNA polymerase locus specifying aphidicolin resistance. Marker transfer experiments showed that the mutation(s) specifying aphidicolin resistance are contained within the EHX-c region of the Aph' mutant DNA. To understand the phenotypic difference of aphidicolin sensitivity between the wild type and the Aph' mutant virus, we determined the nucleotide sequence for this portion.

Figure 3 illustrates the nucleotide sequencing strategy for

FIG. 4. Nucleotide sequence and deduced amino acid sequence of the EHX-c fragment containing the Aph' locus. The 1,147-bp fragment was sequenced by the dideoxynucleotide chain-termination method, and the amino acid sequence was deduced. The nucleotide and amino acid substitutions are boxed. Numbers on the right refer to the last nucleotide or amino acid in each line. Symbols: ●, the nucleotide is different from the one that was present in HSV-1 strain KOS; O, the nucleotides were present in the HSV-2 sequence but were absent in the HSV-1 sequence; underline, the amino acid is different from the one that was present in HSV-1 strain KOS; −3, three nucleotides were deleted in the HSV-2 sequence; parentheses, tandem repeats that were present in the HSV-2 sequence. Restriction sites for *Bam*HI (B), *Eco*RI (E), *Bgl*II (G), *Kpn*I (K), *Pvu*II (P), and *Sal*I (S) are indicated by arrows.

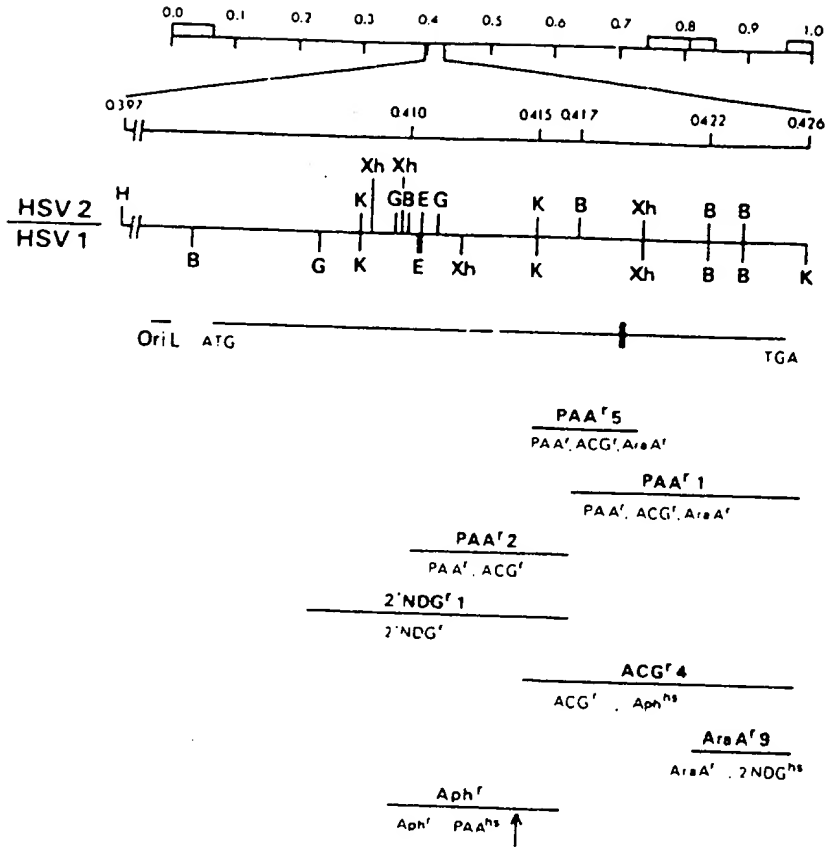


FIG. 6. Map locations of the HSV DNA polymerase locus and the drug-resistant mutations. The numbers on the top line are physical map coordinates of HSV-2 strain 186 DNA. The second and third lines represent the region of HSV-2 DNA between map units 0.397 and 0.426; and the locations of recognition sites for the enzymes *Hin*dIII (H), *Kpn*I (K), *Bgl*II (G), *Bam*HI (B), *Eco*RI (E), and *Xho*I (Xh) are shown above the line for HSV-2 strain 186 and below the line for HSV-1. The fourth line indicates the loci that encode the replication origin (OriL) and the DNA polymerase; the vertical bar shows the locus of highly conserved amino acid sequences among animal DNA polymerases. The locations of mutations in drug-resistant mutants PAA^r5 (7, 9, 15), PAA^r1 (3, 11, 19), PAA^r2 (3, 4, 10) 2′ NDG^r1 (11), ACG^r4 (15), AraA^r9 (15), and Aph^r are indicated at the bottom of the figure. The arrow represents the mutation site of the Aph^r mutant virus that confers aphidicolin resistance.

the EHX-c DNA fragment in which *Eco*RI, *Bgl*II, *Pvu*II, *Sal*I, and *Bam*HI sites were used. Small restriction enzyme fragments were cloned into appropriately cleaved M13 vectors, and single-stranded phage DNAs were used directly as templates for sequence analysis. The DNA sequence of the 1.1-kbp EHX-c fragment within the HSV DNA polymerase gene was thus determined by the dideoxy chain-termination method (Fig. 4). Analysis of the three potential open reading frames of this portion and comparison of this DNA sequence with those recently determined for the DNA polymerase gene of HSV-1 (15, 25) revealed an open reading frame of 1,147 bp that was capable of encoding 382 amino acids. The two EHX-c fragments differed by a single-base change, an AT to GC transition, on the *Kpn*I recognition site of the EHX-c fragment, and this single-base change resulted in the substitution of a tyrosine to a histidine (Fig. 4).

DISCUSSION

Results of this study demonstrates that the Aph^r mutation is physically mapped to a 1.1-kbp region within the DNA polymerase locus of HSV-2. The 1.1-kbp EHX-c fragment (map units 0.410 to 0.417) of the Aph^r mutant also conferred hypersensitivity of a $^{32}P_i$ analog, PAA, to wild-type virus. Although Chiou et al. (5) have shown that mutations within the major DNA-binding protein locus can also confer altered sensitivity to aphidicolin and PAA, our Aph^r mutant did not contain such mutations within the major DNA-binding protein locus. Recently, Gibbs et al. (15) have shown that all of the DNA polymerase mutations conferring altered drug sensitivity from eight different mutants of HSV-1 lie within the 2.5-kbp region between the *Eco*RI site at 0.422 and the *Kpn*I site at 0.434 on the HSV-1 physical map. Our DNA sequence and mapping data indicate that the 1.1-kbp region of HSV-2, which conferred aphidicolin resistance and PAA hypersensitivity, almost overlapped with the 2.5-kbp region of HSV-1; this 1.1-kbp region of HSV-2 corresponded to amino acids 415 to 788 in the predicted amino acid sequence on the HSV-1 *pol* peptide.

The 1.1-kbp DNA sequence of the Aph^r mutant differed from that of the wild type by a single base, and the assignment of the codon reading frame to this region revealed that the mutation changed the codon TAC to CAC, thus changing an amino acid residue of the DNA polymerase protein from a tyrosine to a histidine. The mutation site corresponded to Tyr at residue 696 in the predicted HSV-1

pol polypeptide and was located in a well-conserved region with an amino acid sequence that had extensive homology with the predicted Epstein-Barr virus (EBV) DNA polymerase. According to the alignment of the predicted amino acid sequence for HSV-1 and EBV DNA polymerases by Quinn and McGeoch (25), 41 amino acid residues (starting at amino acid 695) containing the Tyr at residue 696 had 75% homology with the corresponding region of EBV DNA polymerase, while the overall amino acid homology was approximately 39% (Fig. 5). The Aph^r mutation site was exactly located at the left end (*Kpn*I site at 0.415 map units) of the physical map limits of the PAA^r5 and ACG^r4 mutations and was separate from the PAA^r1 and AraA^r9 mutations (Fig. 6). Results of recent studies (14, 15, 25) have revealed the existence of a highly conserved region of 13 amino acids in the carboxyl-terminal end of HSV-1, EBV, adenovirus type 2, and vaccinia virus DNA polymerases. The position of the Tyr-His change, however, was located 185 amino acid residues before the highly conserved region, which began at amino acid 881 on the HSV-1 *pol* peptide. These observations suggest that regions spanning the entire carboxyl-terminal portion of the predicted polymerase polypeptide may contribute to the formation of the binding site(s) for substrates and aphidicolin.

The change of Tyr to His seems to be relatively conservative, but histidine is a basic amino acid and is less hydrophobic than tyrosine (28). When we predicted the secondary structure in the region around the Aph^r mutation site by the method of Chou and Fasman (6), the region around Tyr at residue 286 of wild-type DNA polymerase seemed likely to form a reverse turn flanked by β-sheet structures (Fig. 5), and the substitution to His would be expected to make it relax. Although our data suggest that altered properties of Aph^r DNA polymerase are attributed to a single amino acid change, we cannot at present fully account for the significance of this substitution.

It has been reported that HSV-1 DNA polymerase internally contains two major insertions relative to the predicted EBV DNA polymerase (15, 25), one of which consists of 49 residues starting at residue 646 in the HSV-1 DNA polymerase. The corresponding region (residues 232 to 284 of the EHX-c sequence) of HSV-2 DNA polymerase contained two copies of tandem repeat consisting of 12 nucleotides (4 amino acid residues) and an addition and deletion change and exhibited relatively low homology with the 49-amino-acid region of HSV-1. This region is particularly hydrophilic, so it is likely that it is located on the surface of the HSV DNA polymerase molecule. From these observations, it seems reasonable to speculate that this region may be dispensable for the polymerizing activity, although it lies near the Aph^r mutation site.

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